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- Patents ADP number (if you know it)
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4. Title of the invention METHODS AND MEANS FOR MODULATING APOPTOSIS

5. Name of your agent (if you have one) MEWBURN ELLIS
- "Address for service" in the United Kingdom to which all correspondence should be sent (including the postcode) YORK HOUSE
23 KINGSWAY
LONDON
WC2B 6HP

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METHODS AND MEANS FOR MODULATING APOPTOSIS

The present invention relates in various aspects to methods and means for modulating apoptosis and/or cellular proliferation, in particular via stimulation or inhibition of Fas (also known as APO-1 and CD95). It is based in part on the surprising discovery of anti-Fas autoantibodies in human sera, which antibodies moreover are biologically functional and include both IgG and IgM antibodies. Peptide fragments of Fas and variants and mimetics thereof may be used in modulating apoptosis for therapeutic purposes.

Affinity-purified anti-Fas antibodies isolated from the serum of healthy blood donors have been found by the present inventors to be able to inhibit proliferation and to induce apoptosis of Jurkat leukemia T cells. This effect is inhibited by soluble Fas-Fc chimeric protein. Costimulation of peripheral blood mononuclear cells by human anti-Fas autoantibodies and anti-CD3 monoclonal antibodies induces or inhibits cell proliferation depending on the activation state of the cells. Anti-Fas autoantibodies may thus represent an additional mode of regulation of Fas-mediated signals in vivo which may be harnessed in accordance with the present invention.

25

Fas (also called CD95/APO-1) is a type I cellular receptor protein, belonging to the nerve growth factor/tumor necrosis factor (NGF/TNF) receptor family (Itoh, et al., 1991; Smith

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used in therapeutic contexts including type I diabetes,
multiple sclerosis and liver cirrhosis, and HIV infection.
Tachiban et al (*Cancer Research* (1995) 55: 5528-5530) have
reported correlation between progression of astrocytomas and
5 increased expression of Fas in the tumour cells. De Maria
and Testi (*Immunology Today* (1998) 19: 121-125) review
evidence of cells expressing Fas and its natural ligand in
the proximity of lesions in multiple sclerosis, type I
diabetes, liver diseases and HIV infection.

10

The surprising discovery of anti-Fas autoantibodies in human
serum allows for modulation of binding of those antibodies to
Fas to modulate Fas-mediated effects, particularly apoptosis.
Peptide fragments of Fas, and mimetics thereof, may be used
15 to block antibody binding to Fas, inhibiting or increasing
binding of Fas ligand to Fas. Prior to the work of the
present inventors, it would not have been reasonable to
expect administration of peptide fragments of Fas (a self-
antigen) to have any utility.

20

As noted below in the experimental section, aspects of the
present invention are exemplified by peptide fragments of Fas
known as Fp5, with sequence GQFCHKPCPPGERKARDCTV
corresponding to Gly₄₀-Val₅₉ of Fas, Fp8 with sequence
25 QEGKEYTDKAHFSSKCRRCR, Fp9 with sequence HFSSKRRRCRLCDEGHGLEV,
Fp11, with sequence EINCTRTQNTKCRCKPNFFC corresponding to
Glu₁₀₀-Cys₁₁₉ of Fas, Fp12 with sequence KCRCKPNFFCNSTVCEHCDP,
and Fp17 with sequence WLCLLLLPIPLIVWVKRKEV corresponding to

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embodiments of the present invention include the Fp5, Fp8, Fp9, Fp11, Fp12, Fp17 and Fp18 peptides for which the sequences are provided herein.

5 Experiments show that peptide 16, which has an overlap of 10 amino acids with Fp17, has low or no reactivity with human sera. (Peptide 16 has the sequence KEEGSRNLGWLCLLLLPIP). This provides indication of particular importance for the C-terminal part of Fp17. This is supported by the findings
10 with Fp18 (see Table 1). A further embodiment of the present invention therefore provides a peptide including or consisting of the amino acid sequence QKTCRKHRKE (examples of a peptide including such sequence being Fp17 and Fp18).

15 A peptide for use in the present invention may be a fragment of Fas or may be a variant or derivative thereof, by way of addition, deletion, insertion or substitution of one or more amino acids. Such a variant or derivative thereof will generally retain ability to modulate, either induce or
20 inhibit, apoptosis and/or cellular proliferation (e.g. as measured using Jurkat cells or T-cells).

Preferably, the amino acid sequence of a variant or derivative peptide shares sequence similarity or identity
25 with the relevant Fas fragment sequence, preferably at least about 30%, or 40%, or 50%, or 60%, or 70%, or 75%, or 80%, or 85% similarity or identity, or at least about 90% or 95% similarity or identity. As is well-understood, similarity

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fused to a non-Fas (i.e. heterologous or foreign) sequence, such as a polypeptide or protein domain.

A peptide may provided in isolated form, e.g. after its
5 production by expression from encoding nucleic acid. As noted further below, one or more peptides in accordance with the present invention may be provided by peptide synthesis.

A plurality of peptides each with the amino acid sequence of
10 a different selected peptide may provided in isolated form, individually or in a mixture. Different peptide fragments of Fas that are not naturally joined contiguously (or appropriate variants or derivatives thereof) may be provided joined contiguously together in peptides or polypeptides.

15

A further aspect of the present invention provides the Fp17 peptide, also variants and derivatives thereof that retain the ability to modulate apoptosis and/or cell (e.g. Jurkat or T-cell) proliferation.

20

Peptides and polypeptides (e.g. fusion molecules including a peptide as discussed) in accordance with the present invention may be made using any of a variety of techniques at the disposal of the ordinary person skilled in the art.

25

Peptides may be synthesized using standard peptide chemistry such as by the common method employing Fmoc (Fluorenylmethyl-
ossicarbonil)t-Bu (tert-butyl), as described in Atherton and

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immunization in order to modulate apoptosis and/or cell proliferation in a mammal, such as a human individual for a therapeutic or prophylactic purpose, or a non-human mammal for such a purpose or in order to produce antibodies for
5 subsequent manipulation and/or use (e.g. in diagnostic or therapeutic contexts as discussed further below.)

Nucleic acid encoding a peptide or polypeptide according to the present invention may be used in a method of gene
10 therapy, in modulation of apoptosis and/or cellular proliferation such as in prevention and/or treatment of a disorder in which such modulation has a beneficial effect. This requires use of suitable regulatory elements for expression and a suitable vector for deliver of the
15 expression unit (coding sequence and regulatory elements) to host cells. A variety of vectors, both viral vectors and plasmid vectors, are known in the art, see e.g. US Patent No. 5,252,479 and WO 93/07282. In particular, a number of viruses have been used as gene transfer vectors, including
20 papovaviruses, such as SV40, vaccinia virus, herpesviruses, including HSV and EBV, and retroviruses. Many gene therapy protocols in the prior art have used disabled murine retroviruses. A variety of adenovirus and adeno-associated viral vectors have been developed. Alternatives to viral
25 vectors include transfer mediated by liposomes and direct DNA uptake and receptor-mediated DNA transfer.

Host cells containing nucleic acid encoding a peptide or

and other sequences as appropriate. Vectors may be plasmids, viral e.g. 'phage, or phagemid, as appropriate. For further details see, for example, Molecular Cloning: a Laboratory Manual: 2nd edition, Sambrook et al., 1989, Cold Spring Harbor Laboratory Press. Many known techniques and protocols for manipulation of nucleic acid, for example in preparation of nucleic acid constructs, mutagenesis, sequencing, introduction of DNA into cells and gene expression, and analysis of proteins, are described in detail in Current Protocols in Molecular Biology, Ausubel et al. eds., John Wiley & Sons, 1992.

Systems for cloning and expression of a polypeptide in a variety of different host cells are well known. Suitable host cells include bacteria, eukaryotic cells such as mammalian and yeast, and baculovirus systems. Mammalian cell lines available in the art for expression of a heterologous polypeptide include Chinese hamster ovary cells, HeLa cells, baby hamster kidney cells, COS cells and many others. A common, preferred bacterial host is *E. coli*.

A further aspect of the present invention provides a host cell containing nucleic acid as disclosed herein. The nucleic acid of the invention may be integrated into the genome (e.g. chromosome) of the host cell. Integration may be promoted by inclusion of sequences which promote recombination with the genome, in accordance with standard techniques. The nucleic acid may be on an extra-chromosomal

peptide or polypeptide may be isolated and/or purified from the host cell and/or culture medium, as the case may be, and subsequently used as desired, e.g. in the formulation of a composition which may include one or more additional
 5 components, such as a pharmaceutical composition which includes one or more pharmaceutically acceptable excipients, vehicles or carriers (e.g. see below).

A peptide or polypeptide according to the present invention
 10 may be used as an immunogen or otherwise in obtaining binding antibodies. Antibodies are useful in purification and other manipulation of polypeptides and peptides, diagnostic screening and therapeutic contexts, including passive immunization. This is discussed further below.

15 Particularly useful in such contexts are the Fp17 peptide and variants and derivatives thereof in accordance with the present invention, including fragments of Fp17 including the C-terminal 10 amino acids, and variants and derivatives
 20 thereof.

According to a further aspect of the present invention there is provided a method of obtaining one or more antibody molecules containing a binding site able to bind Fas, the
 25 method including bringing into contact a population of antibody molecules and a peptide according to the present invention, and selecting one or more antibody molecules of the population able to bind said peptide.

constant region or other amino acids, and so on). Instead of using bacteriophage for display, ribosomes or polysomes may be used, e.g. as disclosed in US-A-5643768, US-A-5658754, WO95/11922.

5

Antibody molecules may be provided in isolated form, either individually or in a mixture. A plurality of antibody molecules may be provided in isolated form. Preferred antibodies according to the invention are isolated, in the
10 sense of being free from contaminants such as antibodies able to bind other polypeptides and/or free of serum components. Monoclonal antibodies are preferred for some purposes, though polyclonal antibodies are within the scope of the present invention.

15

The present invention also extends to methods of obtaining and/or raising antibodies to one or more peptides or polypeptides of the invention. Such methods may include administering a peptide or polypeptide or mixture of peptides
20 or polypeptides to a mammal in order to raise an antibody response. In a therapeutic or prophylactic context the mammal may be human or non-human. For the production of antibodies or antibody-producing cells to be isolated and used for any of a variety of purposes, a step of sacrificing
25 a non-human mammal may be included. Such a non-human mammal may be for example mouse, rat, rabbit, dog, cat, pig, horse, donkey, goat, sheep, camel, Old World monkey, chimpanzee or other primate. Antibodies may be obtained from immunized

As an alternative or supplement to immunising a mammal with a peptide, an antibody specific for a protein may be obtained from a recombinantly produced library of expressed immunoglobulin variable domains, e.g. using bacteriophage
5 which display functional immunoglobulin binding domains on their surfaces - for instance see WO92/01047 - or ribosomes/polysomes as noted above. The library may be naive, that is constructed from sequences obtained from an organism which has not been immunised with any of the
10 proteins (or fragments), or may be one constructed using sequences obtained from an organism which has been exposed to the antigen of interest.

Antibodies according to the present invention may be modified
15 in a number of ways. Thus the invention covers antibody fragments, derivatives, functional equivalents and homologues of antibodies, including synthetic molecules and molecules whose shape mimics that of an antibody enabling it to bind an antigen or epitope. Example antibody fragments, capable of
20 binding an antigen or other binding partner are the Fab fragment consisting of the VL, VH, C1 and CH1 domains; the Fd fragment consisting of the VH and CH1 domains; the Fv fragment consisting of the VL and VH domains of a single arm of an antibody; the dAb fragment which consists of a VH
25 domain; isolated CDR regions and F(ab')₂ fragments, a bivalent fragment including two Fab fragments linked by a disulphide bridge at the hinge region. Single chain Fv fragments are also included.

Suitable fluorochromes include fluorescein, rhodamine, phycoerythrin and Texas Red. Suitable chromogenic dyes include diaminobenzidine. Other reporters include macromolecular colloidal particles or particulate material
5 such as latex beads that are coloured, magnetic or paramagnetic, and biologically or chemically active agents that can directly or indirectly cause detectable signals to be visually observed, electronically detected or otherwise recorded. These molecules may be enzymes which catalyse
10 reactions that develop or change colours or cause changes in electrical properties, for example. They may be molecularly excitable, such that electronic transitions between energy states result in characteristic spectral absorptions or emissions. They may include chemical entities used in
15 conjunction with biosensors. Biotin/avidin or biotin/streptavidin and alkaline phosphatase detection systems may be employed.

The mode of determining binding is not a feature of the
20 present invention and those skilled in the art are able to choose a suitable mode according to their preference and general knowledge.

Antibodies according to the present invention may be used in
25 screening for the presence of a peptide or polypeptide, for example in a test sample containing cells or cell lysate as discussed, and may be used in purifying and/or isolating a peptide or polypeptide according to the present invention,

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sample removed from an individual, of cells such as tumour cells expressing high levels of Fas. A method may involving employing an anti-Fp17 antibody (for example), contacting a test sample with the antibody and determining binding of the
5 antibody to the sample.

The present invention also provides assay methods for compounds able to modulate apoptosis and/or cell
10 proliferation.

Further aspects of the present invention provide the use of a peptide of the invention as disclosed, and/or encoding nucleic acid therefor, in screening or searching for and/or
15 obtaining/identifying a substance, e.g. peptide or chemical compound, which interacts and/or binds with the peptide and/or interferes with its ability to bind antibodies directed against it, and/or modulates its ability to affect Fas-mediated apoptosis. Further aspects of the invention
20 similarly provide use of an antibody against a peptide of the invention in screening for a substance able to modulate binding of antibody to the peptide and/or binding of antibody to Fas and/or ability of binding of antibody to Fas to induce or inhibit Fas-mediated apoptosis.

25

For instance, a method according to one aspect of the invention includes providing a peptide or antibody of the invention and bringing it into contact with a substance,

A substance which interacts with the peptide or antibody of the invention may be isolated and/or purified, manufactured and/or used to modulate its activity as discussed.

5 A further aspect of the present invention provides an assay method which includes:

- (a) bringing into contact a substance including a Fas fragment, mutant, variant or derivative thereof, an antibody which is able to bind the substance; and a test compound,
- 10 under conditions in which in the absence of the test compound being an inhibitor, said substance and said antibody interact;
- (b) determining interaction between said substance and said antibody.

15

Such an assay method may determine interaction between complete Fas and antibody, or a Fas fragment, such as a peptide fragment selected from Fp5, Fp8, Fp9, Fp11, Fp12, Fp17, Fp18 and the C-terminal 10 amino acids of Fp17 (N-
20 terminal 10 amino acids of Fp18).

Such an assay may include determination of interaction between Fas and antibody, with a peptide according to the invention also being present, the assay determine the effect
25 of the test substance on ability of the peptide to modulate interaction between Fas and antibody.

The precise format of an assay of the invention may be varied

The amount of apoptosis may be determined, for instance by detecting DNA fragmentation or changes in phosphatidylserine translocation occurring at the cell membrane. DNA fragmentation may be measured using conventional agarose gel electrophoresis. Changes in phosphatidylserine translocation may be analysed by staining with labelled annexin V followed by FACS analysis.

A method of screening for a substance which modulates Fas-mediated apoptosis may include contacting one or more test substances with T-cells or other Fas positive cells and anti-Fas antibodies, particularly antibodies directed against a peptide in accordance with the invention, in a suitable reaction medium, determining the level of apoptosis and comparing that level with the level in a comparable reaction medium untreated with the test substance or substances. A difference in apoptosis between the treated and untreated reaction media is indicative of a modulating effect of the relevant test substance or substances.

20

Combinatorial library technology (Schultz, JS (1996) Biotechnol. Prog. 12:729-743) provides an efficient way of testing a potentially vast number of different substances for ability to modulate Fas activity. Prior to or as well as being screened for modulation of activity, test substances may be screened for ability to interact with a peptide or antibody of the invention, e.g. in a yeast two-hybrid system. This may be used as a coarse screen prior to testing a

only to a substance identified as a modulator of Fas activity, in accordance with what is disclosed herein, but also a pharmaceutical composition, medicament, drug or other composition comprising such a substance, a method comprising 5 administration of such a composition to a patient, e.g. for treatment (which may include preventative treatment) of disease, use of such a substance in manufacture of a composition for administration, e.g. for treatment of disease, and a method of making a pharmaceutical composition 10 comprising admixing such a substance with a pharmaceutically acceptable excipient, vehicle or carrier, and optionally other ingredients.

Also encompassed within the scope of the present invention 15 are functional mimetics of peptide fragments of Fas which are able to modulate apoptosis and/or cellular proliferation. A "functional mimetic" is a substance which may not contain an active portion of the relevant amino acid sequence, and probably is not a peptide at all, but which retains the 20 relevant activity.

Non-peptide "small molecules" are often preferred for many *in vivo* pharmaceutical uses. Accordingly, a mimetic or mimic of the substance (particularly if a peptide) may be designed for 25 pharmaceutical use. The designing of mimetics to a known pharmaceutically active compound is a known approach to the development of pharmaceuticals based on a "lead" compound. This might be desirable where the active compound is

structure of the ligand and its binding partner are modeled. This can be especially useful where the ligand and/or binding partner change conformation on binding, allowing the model to take account of this the design of the mimetic.

5

A template molecule is then selected onto which chemical groups which mimic the pharmacophore can be grafted. The template molecule and the chemical groups grafted on to it can conveniently be selected so that the mimetic is easy to
10 synthesize, is likely to be pharmacologically acceptable, and does not degrade *in vivo*, while retaining the biological activity of the lead compound. The mimetic or mimetics found by this approach can then be screened to see whether they have the target property, or to what extent they exhibit it.
15 Further optimization or modification can then be carried out to arrive at one or more final mimetics for *in vivo* or clinical testing.

Further aspects of the present invention therefore relate to
20 provision of non-peptidyl mimetics of peptides for use in the present invention. One aspect of the invention provides the use of a peptide as disclosed in the identification or design of a non-peptidyl mimetic which retains ability to modulate apoptosis and/or cell proliferation. A further aspect
25 provides a method of testing a non-peptidyl mimetic of a peptide for use in the present invention for ability to modulate apoptosis and/or cellular proliferation.

skill in the art are well able to prepare suitable solutions using, for example, isotonic vehicles such as Sodium Chloride Injection, Ringer's Injection, Lactated Ringer's Injection. Preservatives, stabilisers, buffers, antioxidants and/or
5 other additives may be included, as required.

A peptide may be linked to an appropriate carrier. Various methods of coupling peptides to other molecules are known in the art, including disulphide forming reagents (where the
10 peptide includes a cysteine - or a cysteine is added to the peptide for this purpose), thio-ether forming coupling agents and so on. Carriers include human serum albumin (HSA), tetanus toxoid, other rather large proteins that have reasonable half-lives under physiological conditions, and
15 stable non-proteinaceous molecules such as polysaccharides and copolymers of amino acids.

An adjuvant may be included, such as alum, oil-in-water emulsions or Freund's Adjuvant (Complete or Incomplete).
20 Cytokines may be used to potentiate immunogenicity of the peptide or polypeptide composition.

Whether it is a polypeptide, antibody, peptide, nucleic acid molecule, mimetic, small molecule or other pharmaceutically
25 useful compound according to the present invention that is to be given to an individual, administration may be in a "prophylactically effective amount" or a "therapeutically effective amount" (as the case may be, although prophylaxis

example in a method of making a medicament or pharmaceutical composition including formulating the specific binding member with a pharmaceutically acceptable excipient. Nucleic acid encoding peptides or polypeptides, and non-peptide mimetics
5 may be employed.

A composition may be administered alone or in combination with other treatments, either simultaneously or sequentially dependent upon the condition to be treated and the
10 availability of alternative or additional treatments. In some embodiments of the present invention the relevant molecule is used in conjunction with anti-CD3 antibodies or other anti-CD3 binding molecules.

15 One aspect of the present invention provides use of a peptide as disclosed in the manufacture of a medicament for use in a method of treatment of the human or animal body by therapy. Treatment may be of a proliferative disorder, such as a tumour, cancer or psoriasis, or an autoimmune disorder, type
20 I diabetes, multiple sclerosis, liver cirrhosis and so on. Apoptosis may be induced or inhibited and/or cellular proliferation inhibited or stimulated.

Another aspect provides a method of treating a mammal against
25 such a disorder, the method including administering a peptide or mixture of peptides, mimetic or mimetics, antibody or antibodies or nucleic acid as disclosed, to the mammal.

(open) of Fas-Fc chimeric protein demonstrating that anti-Fas peptide auto-antibodies can induce Fas-mediated apoptosis.

Figure 2(B) shows that anti-Fas peptide auto-antibodies can
5 inhibit apoptosis induced by CH-11 murine anti-Fas antibodies.

Figure 3(A) shows proliferation of T-cell blasts in response
to stimulation with anti-Fas peptide autoantibodies in
10 absence (filled) or presence (open) of immobilized anti-CD3 mAb. The gray bar represents the result of the anti-CD3 mAb alone.

Figure 3(B) shows FasL expression on the surface of PHA-
15 activated T-cell blasts in response to stimulation with anti-Fas peptide autoantibodies in absence (filled) or presence (open) of immobilized anti-CD3 mAb. The gray bar represents the result of the anti-CD3 mAb alone.

20 Figure 4(A) shows proliferation of non-stimulated PBMC in response to stimulation with anti-Fas peptide autoantibodies in absence (filled) or presence (open) of anti-CD3 mAb.

Figure 4(B) shows apoptosis of non-stimulated PBMC in
25 response to stimulation with anti-Fas peptide autoantibodies in absence (filled) or presence (open) of anti-CD3 mAb.

Figure 5 illustrates a molecular model of extracellular

with N-ethyl-N'-(3-dimethylaminopropyl) carbodiimide hydrochloride, as described in the manufacturer's affinity chromatography protocol. After overnight absorption of pooled human sera of healthy blood donors, the anti-peptide
5 antibodies were eluted by 4 M KSCN, concentrated, dialyzed and sterilized by filtration through 0,22 μ m pore size filter. These eluates were analyzed by electrophoresis and Western Blot using NOVEXTM NuPAGE Gel kit: 10% Bis-Tris Gels/MOPS SDS Buffers according to manufacturer's protocols
10 (NOVEX, San Diego, CA). Purified human IgG and mouse IgM, and NOVEX Mark 12TM Wide Range Protein Standard were used as reference.

Solubilized proteins from 10^7 Jurkat leukemia T cells were
15 separated and transferred to 0,45 μ m pore size supported nitrocellulose membrane (Bio-Rad Laboratories AB, Sweden) using NOVEX Western Blot buffer system. Membranes were probed by IgM class anti-Fas mAb CH-11 (Medical Biological Laboratory, Nagoya, Japan) and IgG1 class anti-Fas mAb clone
20 13 (Transduction Laboratories, Lexington, KY) according to manufacturer's recommendations; human control IgG (not reacting with Fas peptides), eluates from affinity columns, pooled human sera before and after column immunoadsorption diluted by 1/50 in PBS containing 5% non-fat dry milk, 0,1%
25 Tween-20 and 0,001% anti-foam agent (Sigma).

Bound antibodies were revealed by sequential use of F(ab)₂ fragments of goat anti-human light chain antibodies

(Pharmacia Biotech, Sweden) for 48 hours. Approximately 5×10^5 stimulated cells were then cultured in a flat-bottom 96-well plate previously coated overnight with anti-Fas peptide affinity isolated antibodies and purified anti-CD3 (OKT-3, 10 $\mu\text{g/ml}$) in RPMI-HEPES medium. After 48 hours incubation, the proliferation was assessed by Alamar Blue Assay (Biosource International, USA.) and the results were expressed as percent difference in reduction of (fluorometric/colorimetric) REDOX indicator between antibody coated and control (without antibodies) wells according to manufacturer's instructions (AMS Biotechnology AB, Sweden).

The FasL expression in PHA- stimulated T-cell blasts was detected at the same time point by use of IgG1 anti-FasL mAb, clone 33 (Transduction Laboratories, Lexington, KY) and Phycoerythrine (PE) conjugated F(ab)₂ fragments of goat anti-mouse IgG (Dako, Sweden). Results were expressed as percent of positive cells analyzed by FACS.

Non-stimulated PBMC were isolated by Ficoll-Hypaque (Pharmacia Biotech, Sweden) and used for proliferation assays as described above for PHA stimulated cells. Apoptosis was quantified by TUNEL assay and expressed as percent difference in fluorescence between antibody containing and antibody-free cultures.

Computer-modelling of Fas

The molecular model for the extracellular domain (Itoh et

subjects.

Similar experiments were performed with commercially available immunoglobulin preparations derived from pooled human serum enriched by immunosorbence for immunoglobulin fractions (Gammagard - Baxter, Hyland, Glendale, CA USA).

Results for the experiments with human serum and with the immunoglobulin preparations are shown in Table 1.

10

The presence of both IgG and IgM auto-antibodies was established.

Autoantibodies to Fas regions represented by Fp11 and Fp17 mediate apoptosis of Jurkat leukemia cells through Fas.

Ability to mediate apoptosis and inhibition of proliferation was determined using antibodies immobilized to plastic surface. Immobilized human antibodies directed to Fp11 and Fp17 induced apoptosis in respectively 49% and 58% of Jurkat leukemia cells (Figure 2(A)). The level of apoptosis induced by Fp5 did not differ significantly from control well in which antibodies were not included, 25% versus 18%.

Next, ability of anti-Fas autoantibodies to affect cell proliferation in the Jurkat cell system was analysed. Human antibodies directed to Fp17 inhibited cell proliferation by 40% as compared to control well; the levels of inhibition of proliferation were lower for anti-Fp5 and anti-Fp11, 8% and

induced by the anti-Fas IgM monoclonal CH-11. Anti-Fas IgG1 murine monoclonal antibodies were previously demonstrated to inhibit the effect of CH-11 (Fadeel et al., 1997). The autoantibodies directed to Fp-5 and Fp-11 reduced CH-11-mediated apoptosis by more than 50%, thus to levels similar to the spontaneous apoptosis occurring in the non-treated cells. Human auto-antibodies to Fp-17 only diminished the CH-11 induced apoptosis by 31% (Figure 2(B)). It has previously been shown that Fp11 can block the apoptotic activity of CH-11 (Fadeel et al., 1995). Thus, the reduction of apoptosis noticed upon incubation with anti-Fp11 is probably due to a specific blocking of the Fas site recognized by CH-11. Antibodies binding to Fp5 and Fp17 of Fas, on the other hand, may sterically hinder binding of CH-11.

Together, these data demonstrate that apoptosis of immortalized Jurkat leukemia T cells induced by human anti-Fas autoantibodies is mediated through a Fas-dependent pathway.

Co-stimulation with anti-CD3 antibodies and anti-Fas autoantibodies affects proliferation according to the state of cell-activation .

Next, ability of human anti-Fas peptide autoantibodies to affect proliferation of primary T cells was investigated. Cross-linking of CD3/TCR receptor is known to trigger a Fas-dependent process termed activation-induced cell death

between 20 and 40% (Figure 4(A)). This effect may be due to reduction of spontaneous apoptosis. Indeed, the costimulation with anti-CD3 antibody and human anti-Fas auto-antibodies induced reduction of spontaneous apoptosis by 15 to 55% for the three anti-peptide antibodies (Figure 4(B)). These data correlate with previous observations that CD3 and Fas costimulation using murine monoclonal antibodies induced increase of proliferation of isolated T cells (Alderson et al., 1993; Alderson et al., 1994).

10

Thus, human anti-Fas auto-antibodies can modulate the biological effect of CD3/TCR ligation in non-stimulated PBMC through a mechanism including reduction of apoptosis.

15 *Fas-binding sites for autoantibodies and Fas L.*

To gain knowledge on how anti-Fas autoantibodies may induce apoptotic/activation signals through Fas, a molecular model of this protein was created where the antibody-binding sites could be visualized in relation to the surfaces of FasL interaction. The extracellular part of the Fas molecule has distinct sequence homology to TNF receptor 1 (TNFR1; Nagata and Golstein, 1995). Based on the three-dimensional structure of TNFR1 a molecular model (Peitsch, 1995 and 1996) of Fas was created (Figure 5).

25

The model shows that antibody binding to the Fas regions represented by Fp5 (Gly₄₀-Val₅₉) and Fp11 (Glu₁₀₀-Cys₁₁₉) may sterically interfere with FasL binding to Arg₈₆ and Arg₈₇,

FasL. The results provide indication that autoantibody binding to this Fp17 region may efficiently transduce a signal to the intracellular cell death machinery.

5 DISCUSSION

The experimental work described above demonstrates the existence of biologically active human autoantibodies against Fas, one of the key molecules in the control of lymphocyte expansion/deletion *in vivo* (Lynch et al., 1995). The anti-Fas autoantibodies were present in the serum of a majority of healthy individuals.

The work also demonstrates that human anti-Fas autoantibodies can trigger cellular responses through the Fas receptor *in vitro*. This provides indication that in addition to already described modes of regulation of Fas-mediated signals (Suda et al., 1993; Irmeler et al., 1997) autoantibodies constitute an additional level of modulation of Fas-mediated functions *in vivo*.

Paralleling previous findings on murine monoclonal antibodies (Owen-Schaub et al., 1992; Klas et al., 1993; Alderson et al., 1993 and 1994), the human autoantibodies against Fas may be used to provide both apoptosis inducing and stimulatory effects depending on the activation state of the cells. Conversion of Fas-mediated signals from cell activation to apoptosis may

therapeutical settings aimed at inducing or reducing Fas-mediated apoptosis.

Measurement of distribution and titres of antibodies
 5 directed to Fas peptides in accordance with the present invention provides further insight into Fas dysregulation and involvement in pathogenesis of diseases. Such distribution and titres are measured in sera obtained from patients with multiple sclerosis,
 10 type I diabetes and HIV infection, using techniques available to those skilled in the art.

Fragments and variants of peptides of the invention which retain ability to inhibit or induce apoptosis are
 15 identified, along with minimal epitopes for binding of anti-Fas antibodies, using systematic substitution in the peptides of each individual amino acid, for instance using alanine scanning. Reactivity of human sera is evaluated with enzyme linked immunosorbent assay to
 20 define amino acids important or necessary for binding.

Colon carcinoma, hepatocellular and other carcinomas in which the expression of Fas is down-regulated (Walker et al. (1997) *J. Immunology* 158: 4521-4524) are treated
 25 with peptides of the invention, particularly Fp17, the C-terminal amino acids of Fp17, and fragments and variants thereof. The portion of Fas including Fp17 includes the membrane spanning peptide and may be used.

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TABLE 1

Reactivity of human serum and immunoglobulin preparations to Fas peptides in ELISA.

| | | | |
|----|----------------|-------------|---|
| 5 | Peptide number | Human serum | Immunoglobulin preparations (Gammagard) |
| | Fp1 | - | - |
| | Fp2 | - | - |
| | Fp3 | - | - |
| 10 | Fp4 | - | - |
| | Fp5 | ++ | + |
| | Fp6 | - | - |
| | Fp7 | - | - |
| | Fp8 | - | +++ |
| 15 | Fp9 | - | ++ |
| | Fp10 | - | - |
| | Fp11 | +++ | +++ |
| | Fp12 | ++ | ++ |
| | Fp13 | - | - |
| 20 | Fp14 | - | - |
| | Fp15 | - | - |
| | Fp16 | - | + |
| | Fp17 | +++ | + |
| 25 | Fp18 | not done | +++ |

+ = <0.5 O.D. 490nm; ++ = <1.0 O.D.; +++ > 1.0 O.D.

M 29.06.99

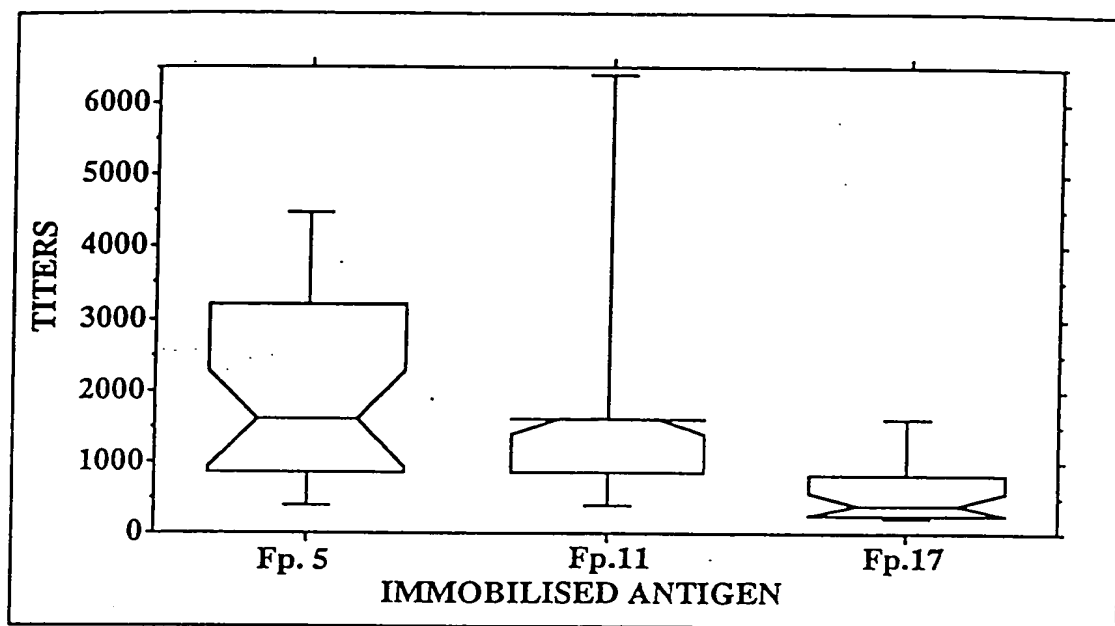


Figure 1

M 29.08.99

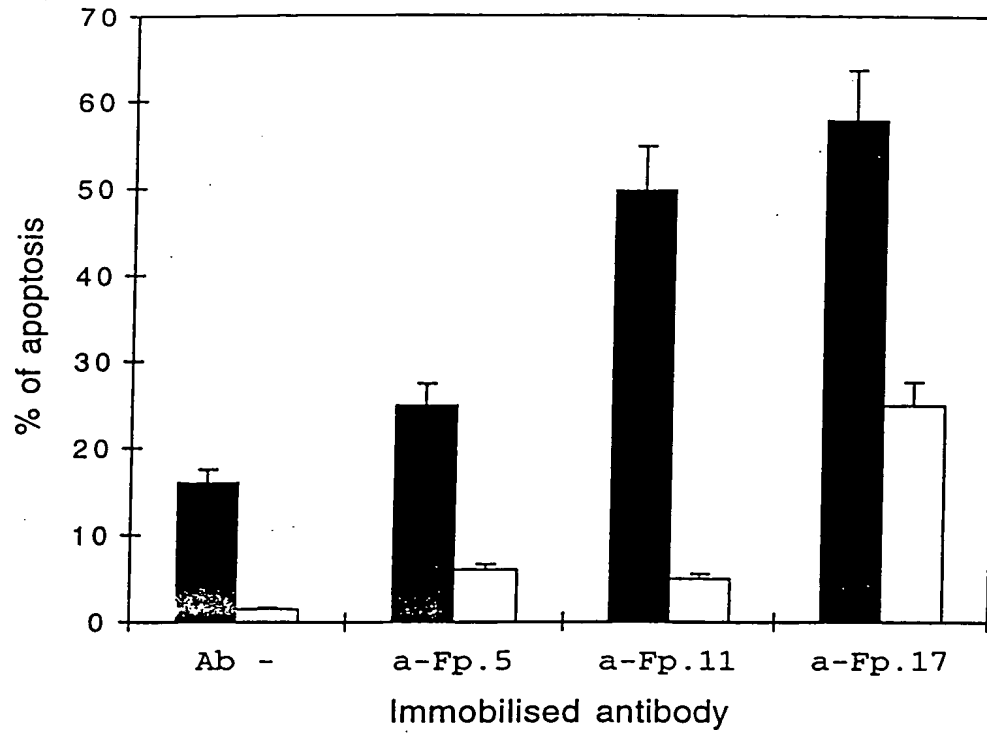


Figure 2a

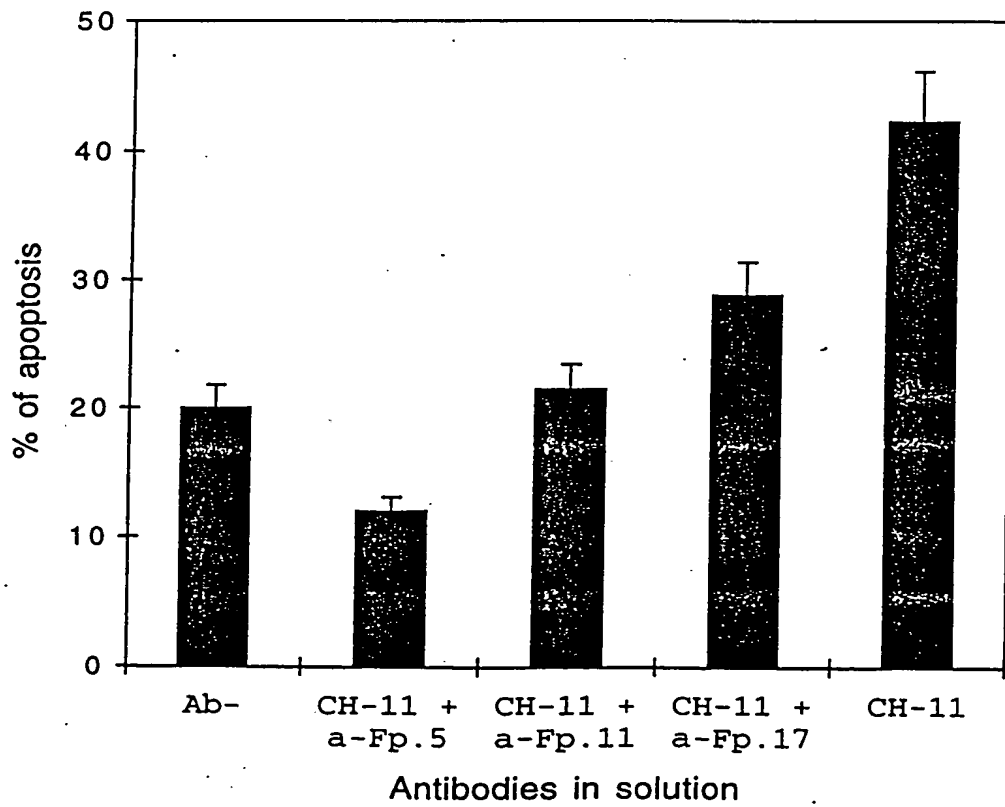


Figure 2b

M 29.06.99

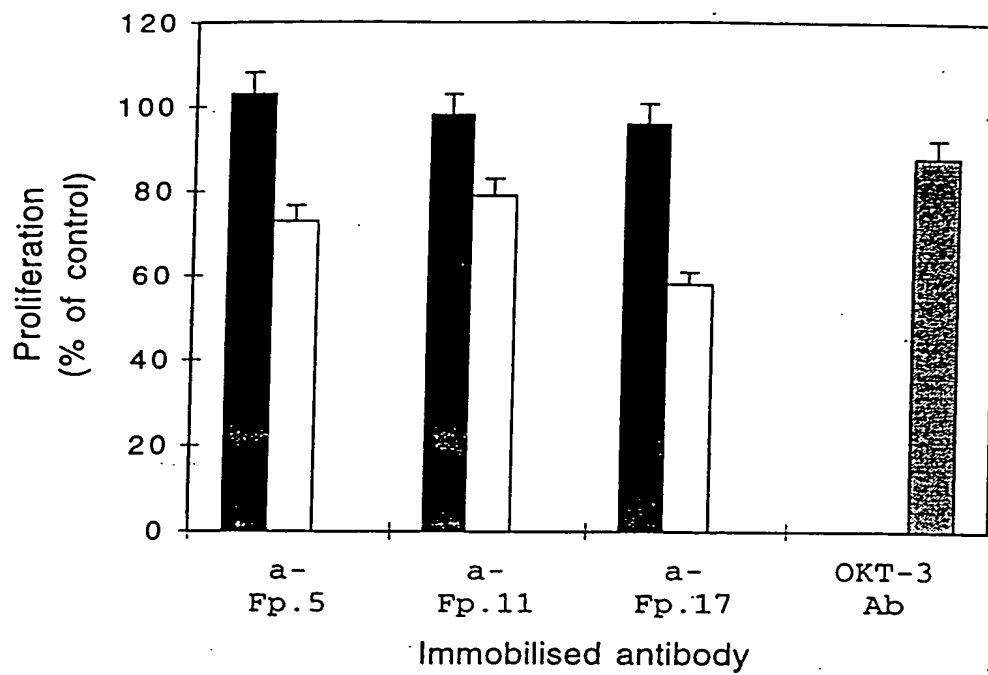


Figure 3a

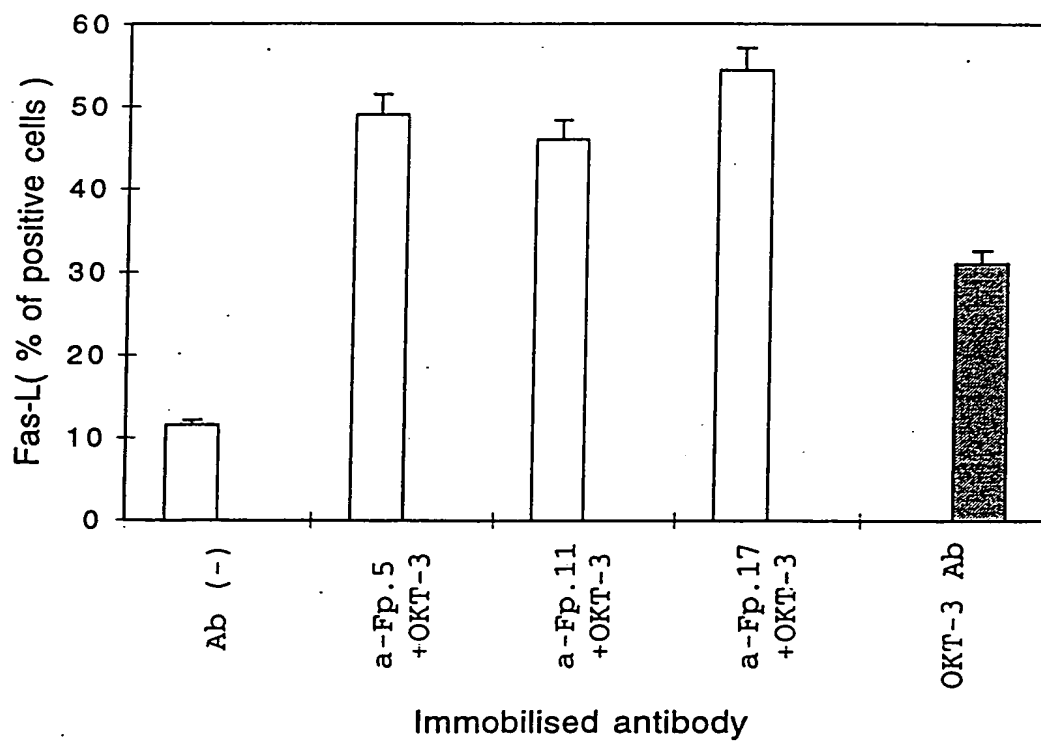


Figure 3b

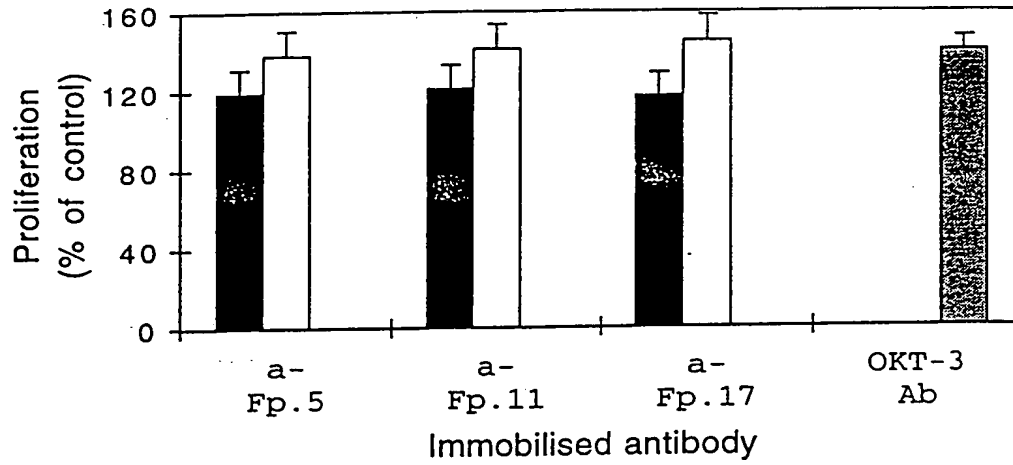


Figure 4a

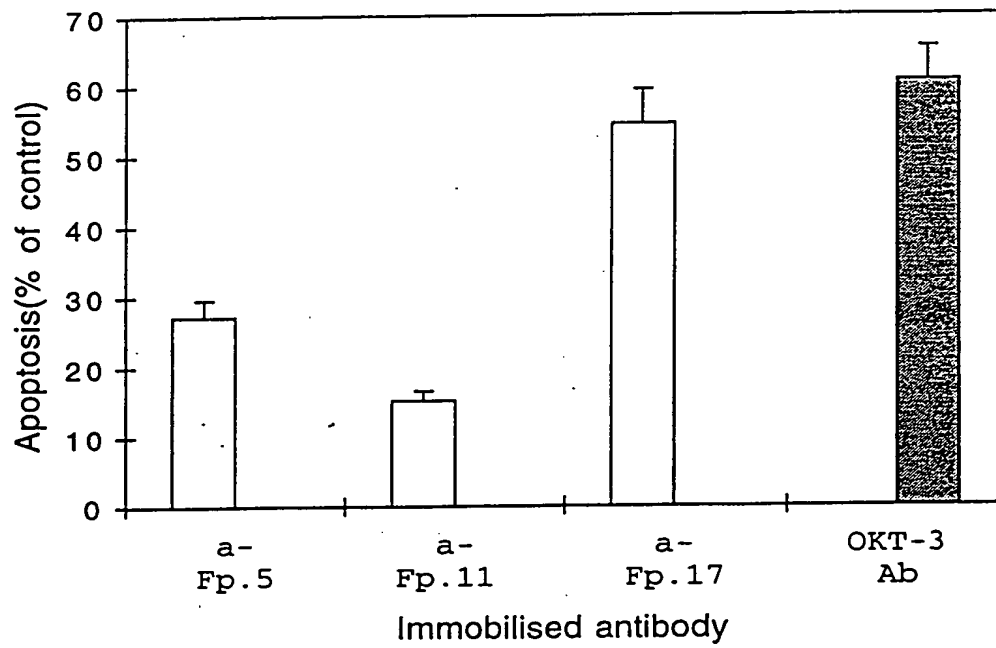


Figure 4b

M 29.06.99

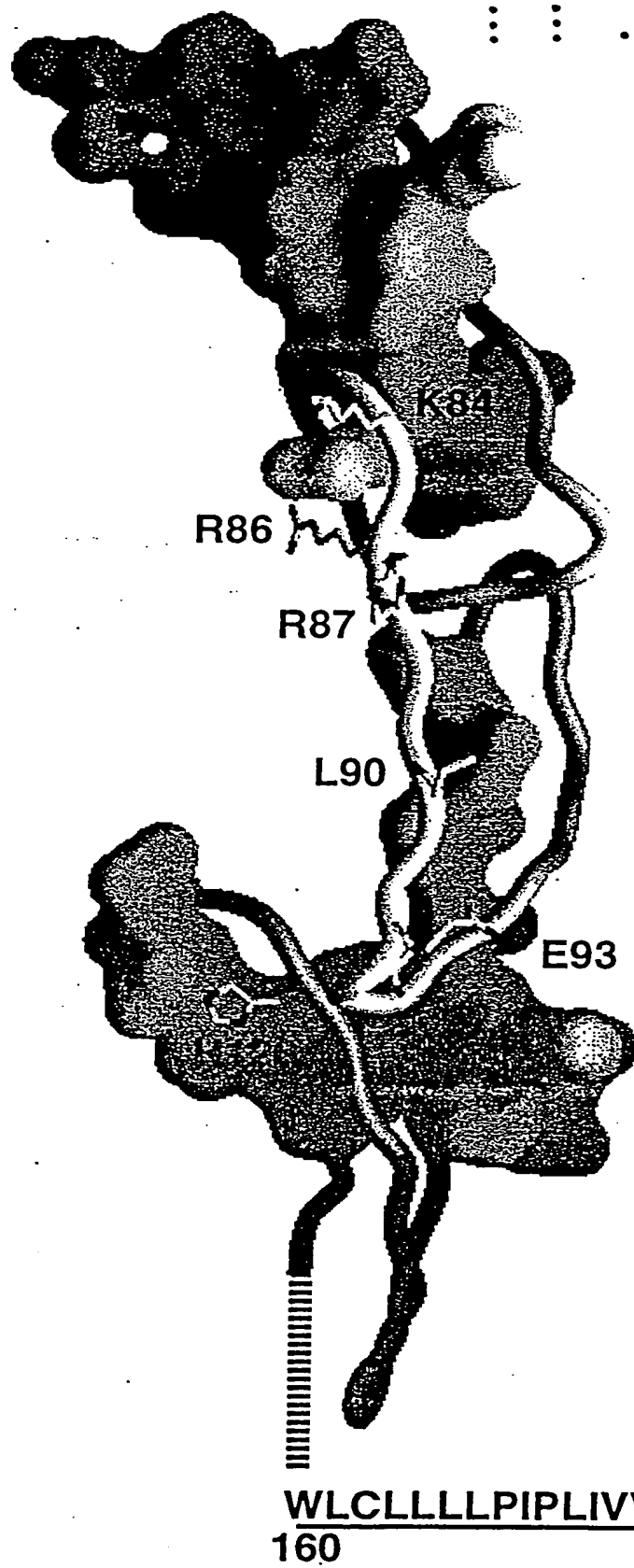


Figure 5